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# Rot1, an essential yeast protein, is degraded through the ER-associated protein degradation system (ERAD)

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**Abstract**

*S. cerevisiae ROT1 is an essential gene which has been related to cell wall biosynthesis, the actin cytoskeleton and protein folding. Rot1 protein is primarily located at the endoplasmic reticulum-nuclear membrane facing the lumen where it is translocated through two internal topogenic elements by an SRP-independent posttranslational mechanism which depends on Sec62 to then be N-glycosylated at the lumen of the ER. Despite Rot1 protein levels are critical for yeast survival, proper cell cycle progression and morphogenesis, Rot1 protein levels has not been investigated so far. Here we carry out an in vivo study to analyse the Rot1 protein levels and show that Rot1 is a short-lived protein and its turnover is mediated by the ubiquitin proteasome system (UPS), dependent on the main degradation pathway located at the ER, the ER-associated degradation system (ERAD). This finding correlates with genetic interactions we previously found and that suggested a link between Rot1 and the ubiquitin-proteasome system. In addition, by using mutant cells components of the ERAD pathway, we demonstrated that Rot1 is degraded through the ubiquitin conjugating enzymes (E2) components of ERAD, Ubc6 and Ubc7, and it seems to require only one of the E3 ubiquitin ligases involved in ERAD, Hrd1 but not Doa1.*

**Keywords:** Rot1, yeast, ER, degradation, ubiquitin ligases, proteasome

## Introduction

Protein levels in cells are governed not only by protein synthesis but also by protein degradation and is critical for maintaining cellular homeostasis. The ubiquitin-proteasome system (UPS) constitutes the major mechanism by which cells acutely alter levels of cytosolic, nuclear, and endoplasmic reticulum (ER) proteins in a highly regulated manner [1, 2]. This occurs generally, but not exclusively, by conjugation with chains of ubiquitin linked through lysine 48 (K48) of ubiquitin, which targets modified proteins to the 26S proteasome (composed by 19S and 20S particles) for degradation [3, 4]. It is worth mentioning that the 20S particle of eukaryotic proteasomes contains several catalytic subunits that hydrolyze peptide bonds with distinct site specificities. The Pre1 subunit is responsible, at least in part, for the chymotryptic activity of the proteasome [5]. Mutations in Pre1 alone, or in combination with mutations in two other proteasomal subunits, Pre2 or Pre4, cause a marked decrease in the rate of degradation of ER-retained proteins [6].

The UPS has been implicated in the degradation of proteins from other organelles, most notably the ER. The ER has garnered the most attention because it requires active protein quality control. A large amount of misfolded or unassembled membrane, secretory proteins as well as ER

luminal proteins are degraded by the ER-associated degradation (ERAD) [7, 8].

Despite ERAD was originally described as quality control system for misfolded proteins, it has wide functions as a protein degradation system [9]. ERAD is conserved in all eukaryote systems and consists of a complex set of E1, E2, E3 processes that are responsible for ubiquitylating and degrading proteins. In budding yeast, ERAD consists of only one E1 enzyme, named Uba1, which is essential for viability [10]. By contrast, it has been found that 11 different E2 enzymes (Ubc1-13) but only three are part of ERAD, Ubc1, Ubc6 and Ubc7 [11]. Concerning E3s there are two well documented, Hrd1/Der3 (which mainly interacts with Ubc7 but also weakly with Ubc1 [12]) and Doa10 (which interacts with Ubc6 and Ubc7). These E3s recognize the specific sequences (commonly known as degron) that initially impact the turnover of the proteins in the cytoplasmic domains (ERAD-C), luminal domain (ERAD-L) o membrane bound ER-proteins (ERAD-M) which are the substrates [13]. The E3 ubiquitin ligase Hrd1, assisted by another subunit named Der1, is involved in targeting proteins located at the lumen (ERAD-L). Hrd interacts mostly with Ubc7 which is recruited to the ER and activated through Cue1. The Cdc48 which is an AAA ATPase is recruited to the ER by the adaptor protein Ubx2. Then, ubiquitylated substrates are cleared out from the ER by Cdc48 and cofactors Ufd1 and Npl4 [9]. The other E3 ubiquitin ligase, Doa10, acts on membrane (ERAD-M) or cytosolic (ERAD-C) ER proteins. In this case, Cdc48 is only required for ERAD-M but not for ERAD-C [9].

Rot1 is an essential protein in the budding yeast *Saccharomyces cerevisiae*, which was identified in a search for second-site suppressors of a *tor2* thermosensitive mutation [14]. Tor2 is involved in growth control and actin cytoskeleton organization. We previously demonstrated that Rot1 protein is involved in several cellular functions as protein folding and cell wall biosynthesis [15, 16], actin cytoskeleton dynamics and cell cycle control [17]. Rot1 is primarily located at the endoplasmic reticulum-nuclear membrane where it is translocated through two internal topogenic elements by an SRP-independent posttranslational mechanism which depends on Sec62 [18, 19]. Rot1 is a membrane protein with unusual topology because the N-terminal of the protein faces the ER lumen, where it is glycosylated, and the single C-terminal hydrophobic region, which contains an essential serine residue at position 250, is essential for proper protein function and cell viability [18–20]. We also found that Rot1 protein genetically interacts with the proteasome [17]. Despite Rot1 cellular levels are critical for yeast survival, how Rot1 gets degraded in cells has not been investigated.

To get deeper insights about how Rot1 protein levels are controlled in cells, we used mutant cells components of the cell degradation system and monitored Rot1 protein levels in shut-off assays. Here we show that Rot1 turnover requires the ubiquitin conjugating E2, Ubc6 and Ubc7, and one of the E3 ubiquitin ligases, Hrd1 but not Doa1 from the ER-associated degradation system (ERAD), and ultimately the *pre1 pre2* subunits of the ubiquitin proteasome system (UPS).

**Results and Discussion**

***The ubiquitin proteasome system plays an essential role in Rot1 protein levels.*** The fact that Rot1 genetically interacts with the proteasome [17, 18] prompted us to investigate whether the UPS could be involved in Rot1 protein degradation. First, Rot1 levels was assayed in promoter shut-off experiments, in which the expression of a *GAL1:ROT1-HA* gene was repressed by adding glucose to the medium in WT cells and the rate of Rot1 degradation was

determined by HA-epitope tagged *ROT1* western analysis. Using this approach, we observed that Rot1 was a short-lived protein. In wild-type cells Rot1 was almost undetectable within 45-60 min after the repression of the *GAL* promoter (Figure 1). In parallel, same assays were performed using cell mutants in the *pre1 pre2* subunits (components of the 20S which impact on the structure of the active site of the proteasome) and transformed with a plasmid containing the gene *GAL1:ROT1-HA*. In contrast to WT cells, Rot1 protein levels poorly decay in the *pre1 pre2* cells bearing the *GAL1:ROT1-HA* plasmid, persisting for more than 120 min after repression of the *GAL* promoter (Figure 1). This result confirmed that Rot1 is degraded through the ubiquitin proteasome system.

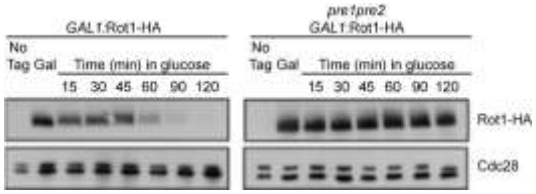
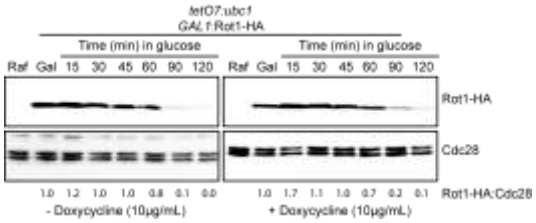



Figure 1. **Analysis of the Rot1 protein levels in the mutant strain in the proteasome subunits *pre1pre2*.** Cells of wild type (WCG4α) and mutant *pre1 pre2* (WCG4-11/22) transformed with a plasmid that contains the Rot1-HA protein under GAL1 promoter were grown in SD Gal overnight and then transferred to glucose medium. The levels of the Rot1 protein Rot1 after the repression of the *GAL*1 promoter by glucose were analyzed by Western blot at the indicated times. A cell extract obtained from the wild strain (W303) is included as negative control (no tag). The level of protein Cdc28 is shown as loading control.

Similar results were obtained when expressing *GAL-ROT1-HA* endogenous or ectopically using a plasmid we previously generated [18], indicating that expressing of Rot1 does not change its stability in cells (data not shown). Same decay kinetics was also obtained when performing translational shut-off experiments carried out with the same cells to follow Rot1 decay after addition of cycloheximide by western blot (data not shown).

***Turnover of Rot1 proceeds via the two E2 proteins from ERAD, Ubc6 and Ubc7*** Rot1 is a transmembrane protein primarily located at the endoplasmic reticulum (ER) facing the lumen [18]. Since the prevalent pathway for ER-membrane protein turnover is ERAD, we hypothesized that ERAD could mediate Rot1 degradation. We performed shut-off experiments in the three documented ubiquitin conjugating enzymes (E2s) yeast mutant cells (Ubc1, Ubc6 and Ubc7) components of the ERAD pathway [2, 9] while expressing the plasmid containing the gene *GAL1:ROT1-HA*.

Because *UBC1* gene is essential for viability, we used a conditional mutant strain that expresses the *UBC1* gene under the control of the doxycycline-regulated *tetO7* promoter (*tetO7:UBC1*). In cells growing in the presence of 10 µg/mL doxycycline, i.e. deficient in *UBC1*, and after *GAL1:ROT1-HA* repression by glucose to the culture cells, we observed that the rate of Rot1 degradation was indistinguishable from control cells. This suggested that Rot1 degradation is not Ubc1-dependent (Figure 2). Next, we studied the other two E2 enzymes involved in ERAD: Ubc6 and Ubc7. We compared Rot1 degradation in *ubc6* and *ubc7* cells versus WT cells. Both mutants show similar Rot1 levels, being detectable by western blot throughout the two-hour experiment (Figure 3). These results suggest that Ubc6 and Ubc7 are regulating Rot1 protein




levels.

Figure 2. **Analysis of the stability of the protein Rot1 in the mutant strain in the E2 enzyme Ubc1**. Cultures of the mutant strain *tetO7: ubc1* transformed with a plasmid containing the Rot1-HA protein under the *GAL1* promoter were grown in the presence or absence of doxycycline (10µg / mL) and in raffinose (Raf) as a carbon source. Then galactose was added to the medium (Gal) during 1h and cells were transferred to medium with glucose. The levels of the Rot1 protein after repression of the *GAL*1 promoter were analyzed by Western blot at the indicated times. The level of protein Cdc28 is shown as loading control. Below each lane of the blot is the ratio of Rot1 to Cdc28 relative to the first time point in Gal (t=0 min).

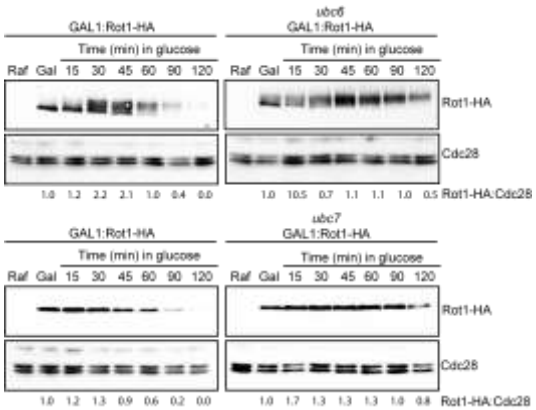



Figure 3. **Analysis of the stability of the protein Rot1 in the mutant strains in the enzymes E2: Ubc6 and Ubc7**. Cultures of the yeast mutant strains *ubc6* and *ubc7* transformed with a plasmid containing the Rot1-HA protein under the *GAL1*promoter were grown in raffinose (Raf) as a carbon source. Then galactose was added to the medium (Gal) during 1h and cells were transferred to medium with glucose. The levels of the Rot1 protein were analyzed by Western blot at the indicated times. The level of the Cdc28 protein is shown as loading control. Below each lane of the blot is the ratio of Rot1 to Cdc28 relative to the first time point in Gal (t=0 min).

We wondered if the two E2 activities could be synergizing Rot1 degradation in cells. To test this, we performed similar shut off experiments using the *ubc6 ubc7* double mutant. As expected in WT cells, Rot1 levels are basically undetectable after 45-60 min of *GAL1* promoter repression (Figure 4). In sharp contrast, *ubc6 ubc7* mutant cells Rot1 protein levels barely decay during the 2 hours of the experiment (Figure 4).

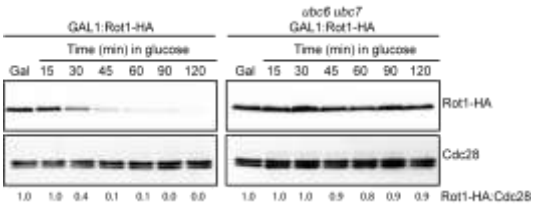



Figure 4. **Analysis of the stability of the protein Rot1 in the double mutant in the enzymes E2: Ubc6 / Ubc7**. Cultures of the yeast mutant strains *ubc6ubc7* transformed with a plasmid containing the Rot1-HA protein under the *GAL1* promoter were grown in raffinose (Raf) as a carbon source. Then galactose was added to the medium (Gal) during 1h and cells were transferred to medium with glucose. The levels of the Rot1 protein were analyzed by Western blot at the indicated times. The level of the Cdc28 protein is shown as loading control. Below each lane of the blot is the ratio of Rot1 to Cdc28 relative to the first time point in Gal (t=0 min).

We noticed that the Rot1's protein pattern was slightly different in *ubc6* than in *ubc7* cells. It presented some smearing and diffuse band pattern in absence of *UBC6*. Then, these results show that Rot1 turnover is mediated by both E2, Ubc6 and/or Ubc7 and these enzymes may teamwork sequentially to achieve proper Rot1

degradation. It could be plausible that Rot1 gets mono-ubiquitylated by Ubc6 and triggers Rot1 ubiquitylation by Ubc7 to enhance Rot1 degradation.

***The ERAD E3-ubiquitin ligase Hrd1 plays a role in Rot1 degradation.*** In budding yeast, there are at least two well-known E3-ubiquitin ligase associated to the ERAD pathway, Hrd1/Der3 and Doa10 [9]**.** Following same strategy than for the E2 study, we checked Rot1 degradation in cells lacking those E3-ubiquitin ligase. We monitored Rot1 levels in the *hrd1 doa10* double mutant (Figure 5). Notably, Rot1 levels were stabilized yet highly diffused in the E3-ubiquitin ligase double mutant cells, a pattern never detected in WT cells. The shift observed in Rot1 mobility could be attributed to Rot1 other posttranslational modifications such as glycosylation or phosphorylation.

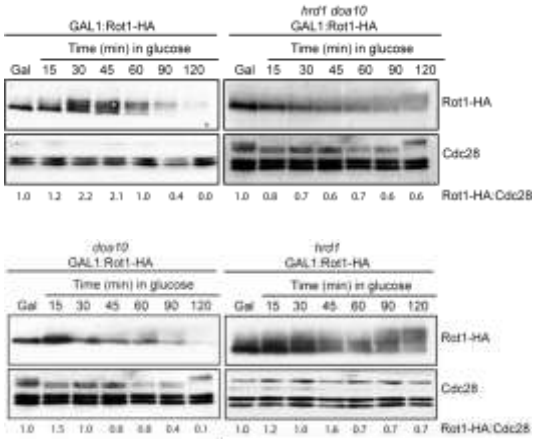



**Figure 5. Analysis of the stability of the protein Rot1 in the double mutant in the enzymes E3: Hrd1 / Doa10 and the individual mutant strains Hrd1 and Doa10.** Cultures of the yeast mutant strains *hrd1doa10* transformed with a plasmid containing the Rot1-HA protein under the *GAL1*promoter were grown in raffinose (Raf) as a carbon source. Then galactose was added to the medium (Gal) during 1h and cells were transferred to medium with glucose. The levels of the Rot1 protein were analyzed by Western blot at the indicated times. The level of the Cdc28 protein is shown as loading control. Below each lane of the blot is the ratio of Rot1 to Cdc28 relative to the first time point in Gal (t=0 min).

In order to determine whether one or both E3 contributes to Rot1 degradation, same experimental approach was performed but using the *hrd1* or *doa1* individual mutant cells. The results obtained suggest that Hrd1 could be the only E3-ubiquitin ligase promoting Rot1 degradation because we could not observe any appreciable change in Rot1 degradation rate in the *doa1* mutant cells versus WT cells (Figure 5). Moreover, *hrd1* mutant cells showed a shift in Rot1 levels similar than that observed in the *hrd1 doa10* double mutant, pointing that the protein gets ubiquitylated by not properly degraded in absence of Hrd1.

Then, a possible scenario could be that Rot1 is degraded by ERAD (Figure 6). On one side, the chief ubiquitin-conjugating enzymes E2, Ubc6 and Ubc7, are required for Rot1 degradation either in a cooperative or redundant manner. The resulting ubiquitin-conjugating E2-Rot1 complex is then processed at the ER by the E3-ubiquitin ligase Hrd1 but not by Doa1. Interestingly, Hrd1 is responsible of degrading misfolded intramembrane proteins (ERAD-M) or proteins that localize in the lumen (ERAD-L), by contrast to Doa1 that targets misfolded cytosolic domain of proteins around the ER (ERAD-C) [21]. Then, this agrees with Hrd1 targeting Rot1, which faces the lumen of the ER, for degradation. However, we believe that during the course of our experiments Rot1 protein is kept in its native form and not misfolded. Thus, these results claim the question whether Hrd1 is capable to degrade a broader spectrum of proteins that has been assumed so far. In short, our findings reveal that Rot1 is a protein with a short half-life

and is degraded via ERAD but also open a new avenue for further investigations about the plethora of substrates that Hrd1 E3-ubiquitin ligase targets in cells.

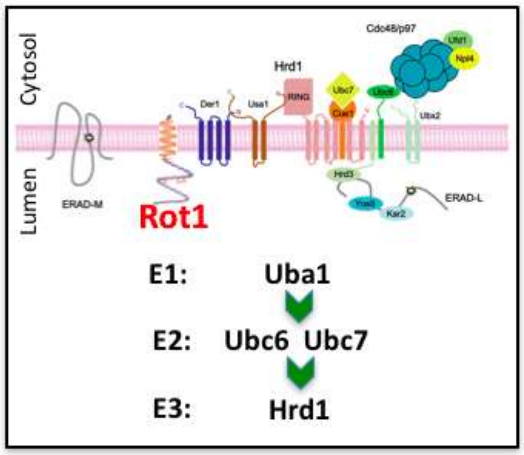



**Figure 6. Schematic of Rot1 domains and ERAD machinery located at the ER.** Below the schematic of the E1, E2, E3 identified as required for Rot1 degradation.

## Materials and methods

**Yeast Strains, Genetic Methods, and Plasmids.** All ERAD yeast strains were derivatives of W303 (*ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3, ssd1*) kindly provided by M. Hochstrasser. Wild-type (WCG4α) and mutant *pre1pre2* (WCG4-11/22) obtained generously from S.D. Wolf. The strain *tetO7:UBC1* gene was acquired from *Open Biosystems.* Cells were grown on standard yeast synthetic dextrose (SD), or rafinose (SRaf) or galactose (SGal) medium supplemented as required. Doxycycline (Sigma) was added at a concentration of 10 µg/mL to fully repress the *tetO7* promoter. Yeast strain GAL1:ROT1-HA in W303 background and plasmid pGAL:ROT1-HA in which the *ROT1* promoter was substituted for the *GAL1* promoter either in the genome or in the plasmid pROT1-HA-CEN (centromeric and encoding three copies of the HA epitope integrated in frame in the middle region of the protein) have been previously described in [18].

**Rot1 protein stability assays.** To evaluate the Rot1 protein decay, WT strains and the different mutant cell studied carrying the p*GAL1:ROT1-HA* plasmid were grown directly on galactose synthetic medium (SGal) or in rafinose (SRaf). Then, to repress the *GAL1* promoter, glucose to a final concentration of 2% was added to the media. Cells were collected at different times during 120 min and processed for Western blot analysis. To evaluate the Rot1 protein decay in *ubc1* mutant cells (*tetO7:UBC1* gene), cells were incubated in the presence of 10 µg/mL doxycicline for at least 6 hours to shut off *UBC1* gene expression and then glucose was added to repress *GAL1* promoter as above indicated.

**Western blot analysis.** Approximately $10^8$ cells were collected, resuspended in 100 µL of water, and after adding 100 µL of 0.2 M NaOH, they were incubated for 10 min at room temperature. Cells were collected by centrifugation, resuspended in 50 µL of sample buffer (150 mM Tris-HCl (pH 6.8), 300 mM DTT, 6% SDS, 0.3% bromophenol blue, and 30% glycerol) and incubated for 5 min at 95ºC. Extracts were clarified by centrifugation, and equivalent amounts of protein were resolved by SDS-PAGE. After transfer to nitrocellulose filters, tagged proteins were detected with the corresponding antibody (anti HA 3F10 rat monoclonal antibody from Roche, dilution 1:1000) for western detection of Rot1-HA, anti-PSTAIRE antibody (Santa Cruz) for western detection of Cdc28, dilution 1:1000) and the ECL Advance Western blotting detection Kit (GE Healthcare) following the manufacturer's instructions

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